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Award Number: DAMD17-99-1-9276

TITLE: Role of Whn in Mammary Gland Development and Tumorigenesis

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REPORT DATE: October 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2000	Annual Summary (30 Sep 99 - 29 Sep 00)

4. TITLE AND SUBTITLE
Role of Whn in Mammary Gland Development and Tumorigenesis

5. FUNDING NUMBERS
DAMD17-99-1-9276

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
This report contains colored photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; Distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

We are studying the function of Whn, a winged helix/forkhead transcription factor, in mammary gland development and tumorigenesis. Previous work from our laboratory investigating Whn function in the skin has shown that it has roles in both proliferation and differentiation. Consistent with this data, mice lacking functional Whn have defects in the formation and function of the mammary epithelia. We have shown that Whn is expressed in nuclei of mammary epithelial cells, and that it is present at times when the gland is undergoing periods of proliferation and differentiation. Transgenic mice have been created in which Whn is targeted to the mammary epithelium by use of the mouse mammary tumor virus (MMTV) promoter. These mice will be used to characterize the effects of increased Whn expression on mammary gland development and function. Investigation of Whn in human breast cell lines has indicated that the protein is expressed at low levels in normal breast cells but may be decreased or lost in some tumor cell lines. This indicates that loss of the protein, rather than a gain of function, may be involved in the malignant progression.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
17

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Introduction

Mice that lack functional Whn (nude mice) have defects in the development of the mammary glands, in addition to the well characterized skin and thymus defects (Sundberg, 1994). Whn is a member of the winged helix/forkhead family of transcription factors and is present in both rodents (Kurooka et al., 1996; Nehls et al., 1996; Nehls et al., 1994; Schorpp et al., 1997; Segre et al., 1995), and humans (Frank et al., 1999). We are studying the role of Whn in normal mammary gland development and tumorigenesis since the function of this protein in the mammary gland is unknown. Previous work from our laboratory investigating Whn function in the skin has shown that it has roles in both proliferation and differentiation (Baxter and Brissette, 2000 ; Lee et al., 1999; Prowse et al., 1999). Consistent with this data, we have shown that nude mice have defects in the initial development of the epithelial network of ducts when the tissue is undergoing a period of intense proliferation. In addition, the nude mouse mammary glands fail to develop normally during pregnancy suggesting that Whn is necessary for the mammary epithelial cells to correctly differentiate. To further investigate the requirements for Whn in the mammary gland, transgenic mice have been created in which Whn is overexpressed in the mammary epithelium. Mammary gland development will be compared between these mice, wild-type and nude mice. In addition, the transgenic mice will be monitored for tumor formation because it has been shown that overexpression of Whn in the epidermis leads to overproliferation (Prowse et al., 1999).

Body

Task 1. Determine the temporal and spatial expression of Whn during mammary gland development

Whn is expressed in mammary epithelial cells

Initial experiments showed that mouse mammary glands lacking Whn function have proliferation and differentiation defects, similar to those observed in the nude mouse epidermis. We found that the growth of the ductal tree in the fat pad of the virgin nude mice is retarded, and the degree of branching is significantly reduced compared to wild-type controls, suggesting that Whn is required for epithelial proliferation. At late pregnancy, the nude glands are significantly smaller, approximately half the size of wild-type, with branches that are densely packed. Immediately following birth, the lumens of the nude glands remain filled with premature secretions, similar to that seen in the wild-type at late pregnancy, despite the presence of pups attempting to suckle. The epithelia of the nude alveoli are

thinner than the wild-type, and in some cases the myoepithelial layer appears to be discontinuous. These data suggest a defect in the late stages of differentiation.

To investigate the *whn* expression pattern in mammary gland development we utilized transgenic mice in which an endogenous *whn* gene has been disrupted by a β-galactosidase (*lacZ*)-neomycin cassette (Nehls et al., 1996). This targeted insertion fuses the *whn* promoter to *lacZ* and thus β-galactosidase activity serves as a marker of *whn* transcription. While the insertion inactivates the *whn* gene, this mutation is recessive, and therefore heterozygous mice are morphologically and functionally normal (Nehls et al., 1996). Mammary glands were removed from pregnant mice heterozygous for the insertion and stained for β-galactosidase activity. Glands from wild-type mice were included as a negative control. Under a variety of different fixation and staining conditions we could not identify staining that was specific to the transgenic mice. Consultation with other scientists who have analyzed β-galactosidase activity in mouse mammary glands confirmed that this tissue commonly shows high background activity.

Therefore we employed immunohistochemistry to determine the cell types expressing Whn. Sections of formaldehyde-fixed paraffin-embedded mammary tissue from pregnant wild-type and nude mice were stained with polyclonal antibodies raised against full-length murine Whn protein. Figure 1 (Appendix 1) shows that, as expected, Whn is present in the nuclei of the epithelial cells surrounding the alveoli of the wild-type mammary glands. The nude tissue served as a negative control for the antibody staining.

The Whn expression pattern during mammary gland development correlates with periods of active growth and differentiation.

To determine the times at which *whn* is expressed in the mammary gland, reverse transcriptase-mediated polymerase chain reaction (RT-PCR) was performed on RNA extracted from wild-type mammary glands at various stages of development. cDNA was prepared from RNA by incubation with reverse transcriptase and random oligonucleotide primers. The presence of *whn* transcript was determined by PCR using oligonucleotide primers specific for *whn*. Preliminary results in figure 2 (Appendix 1) show that *whn* is expressed in the four week old virgin gland, but not in the glands taken from seven and ten week old mice. In a four week old mouse the mammary gland is undergoing a period of intense proliferation as the terminal end buds appear and the epithelial ducts grow out into the mammary fat pad. This process is usually complete by about six weeks of age. Thus the expression of *whn* at four weeks, but not at seven or ten weeks, indicates that Whn is involved in epithelial proliferation.

During pregnancy, the mammary glands develop further and differentiate to form secretory lobuloalveoli that produce milk for the pups. *Whn* expression is seen in the mammary glands taken from pregnant mice, starting at day 15 and continuing through two days postpartum. These times correspond to the period when the tissue is actively growing and differentiating. Oligonucleotide primers specific for *β-actin*, a gene that is constitutively expressed, were used as a positive control to ensure the integrity of the RNA (Tokunaga et al., 1986). Figure 2 (Appendix 1) shows that *β-actin* is present in all samples, even when *whn* was not detected. Reactions in which the reverse transcriptase enzyme was omitted were negative for both *whn* and *β-actin* (data not shown). Consistent with data on the function of Whn in the epidermis (Baxter and Brissette, 2000 ; Lee et al., 1999; Prowse et al., 1999), these data indicate that Whn is involved in both proliferation and differentiation of the mammary epithelium.

Abnormalities in nude mouse mammary gland development are due to intrinsic defects of the mammary epithelial cells

To unambiguously establish that the abnormalities in nude mouse mammary gland development are due to defects in the epithelial cells and not hormonal, estrogen and progesterone levels were measured in serum taken from nude and wild-type mice during development and pregnancy. There was no significant difference in the levels of these hormones in either the developing virgin mice or during pregnancy. These data coupled with the temporal and spatial expression patterns of Whn support the hypothesis that the expression of Whn is required in mammary gland formation and differentiation, and that the nude phenotype is due to intrinsic defects of the epithelial cells and is not the secondary consequence of reduced steroid hormone levels.

Whn is not required for the transcription of β-casein, α-lactalbumin, and whey acidic protein

Since nude mice fail to lactate and Whn is a transcription factor, we investigated whether it has any effect on the transcription of milk protein genes. RNA was extracted from mammary glands taken from wild-type and nude mice during pregnancy and following the birth of the pups, and the levels of β-casein (Robinson et al., 1995), α-lactalbumin (Robinson et al., 1995; Vilotte and Soulier, 1992) and whey acidic protein (WAP) (Pittius et al., 1988; Robinson et al., 1995) were determined by northern blotting. Although the nude mothers cannot nourish their pups, figure 3 (Appendix 1) shows that the transcription levels of these three milk proteins are the same in the glands from the nude mice as they are in wild-type. Thus, Whn is not required for the transcription of these milk proteins. Since the production of the milk proteins is under hormonal control (Rosen, 1987; Topper and Freeman, 1980) the fact that Whn is not required for their production is further evidence for the nude mammary defect being intrinsic to the mammary epithelial cells.

Task 2. Create and analyze transgenic mice that overexpress Whn in mammary epithelia

To target *whn* to the mammary epithelia, we generated a construct that places *whn* under the control of the mouse mammary tumor virus (MMTV) promoter (Cardiff, 1996; Stewart et al., 1984). This promoter contains all the necessary elements to target expression to the mammary epithelia and the construct consists of the complete mouse *whn* cDNA containing a FLAG epitope tag (Knappik and Pluckthun, 1994) at the translational start site, enabling us to distinguish ectopically-expressed Whn from the endogenous protein. The *MMTV-whn* construct was injected into fertilized eggs of C57BL6 x DBA mice, and the presence of an intact transgene in the resulting pups was determined by PCR and Southern blotting (figure 4A and B (Appendix 1)). In total 18 founder mice were positive for the *MMTV-whn* transgene, and 12 of these transmitted it to the next generation. Transgene expression was examined by immunoprecipitating Flag-tagged Whn from total mammary gland extracts, followed by separation on SDS-PAGE and immunoblotting with antibodies specific for Whn. Figure 4C (Appendix 1) shows an immunoblot for a representative *MMTV-whn* transgenic mouse and a control mouse that does not express the FLAG tagged protein.

To investigate the effects of Whn overexpression on early mammary gland development whole mounts were prepared from transgenic mice and wild-type litter mates. In an initial analysis, no differences in morphology were observed between the transgenic mice and their wild-type litter mates. In order to increase the levels of overexpression, four lines of *MMTV-whn* transgenic mice were chosen that had the highest levels of transgene expression, and siblings are being mated in order to generate mice homozygous for the transgene. These mice will be used to further investigate the effects of Whn overexpression in the mammary epithelia.

Biochemical characterization of Whn

Analysis of Whn in normal mouse mammary tissue has indicated that, like other transcription factors, the protein is expressed at very low levels. Mammary glands are derived from epidermis (Sakakura, 1987; Taylor-Papadimitriou and Lane, 1987), and the skin and mammary gland show many similarities, for instance the expression of certain keratins (Smith et al., 1990; Taylor-Papadimitriou and Lane, 1987), and the requirment for mesenchymal epithelial interactions in development (Cunha, 1994; Gouon-Evans et al., 2000). Murine keratinocytes can be grown in culture and induced to differentiate in a way that reflects the changes that take place in the epidermis *in vivo* (Hennings and Holbrook, 1983; Hennings et al., 1980). Under differentiating conditions the levels of Whn significantly increase as keratinocytes initiate terminal differentiation, therefore we have used this *in vitro* model system to investigate biochemical changes in Whn during differentiation. Whn protein isolated from keratinocytes resolves as multiple bands on SDS-PAGE gels, indicating that it may undergo posttranslational modification. Treatment of the protein with phosphatase reduces the multiple species to a single, faster migrating band, while inclusion of a

phosphatase inhibitor in the reaction prevents the phosphatase from altering the mobility of the Whn protein. Phosphorylation of Whn was confirmed by metabolically labeling keratinocytes with ^{32}P-inorganic phosphate followed by immunoprecipitation with Whn specific antibodies and separation on SDS-PAGE. Phosphopeptide mapping shows that Whn is phosphorylated at several site and phosphoamino acid analysis indicates that the primary site of phosphorylation is at serine residues. Initial data show that while the total amount of Whn is modulated during differentiation, the phosphorylation state remains unaltered (Baxter and Brissette, 2000). Simillarly the Whn protein found in mammary glands also appears to undergo posttranslational modification.

Task 3. Analysis of Whn protein in human breast cancer sections and cell lines.

The human and murine Whn proteins are highly conserved (85% identical) (Schorpp et al., 1997), which strongly suggests that Whn will play a similar role in the two species. The human gene maps to chromosome 17q (Schorpp et al., 1997; Segre et al., 1995), a region frequently amplified in human breast cancer (Bieche et al., 1996; Guan et al., 1994; Kallioniemi et al., 1992; Muleris et al., 1994; Tomasetto et al., 1995), and overexpression of Whn in the epidermis leads to a hyperproliferative phenotype (Prowse et al., 1999). Therefore, we have investigated the expression of *whn* in human breast cancer cell lines. A cell line derived from normal human mammary epithelium and several lines derived from breast tumors were obtained from the American Type Culture Collection. Cell lysates were prepared and subjected to immunoprecipitation with Whn specific antibodies. Levels of Whn were determined by immunoblotting the precipitated proteins. Figure 5 (Appendix 1) that in an initial immunoblot Whn was detected at low levels in the normal breast cell line, HS 578 BST, and in several of the tumor cell lines. These data show that Whn expression is highest in the normal breast cell line which suggests that the loss or decrease of Whn expression may be involved in malignant progression. This is in contrast to the situation seen in the epidermis where overexpression of Whn resulted in increased proliferation. However, these results may be explained by the further observation that Whn promotes the differentiation of the cell in which it is expressed (Baxter and Brissette, 2000). Thus the loss of Whn function may result in a cell that has increased proliferative potential and can contribute to the formation of poorly differentiated tumors.

Key research accomplishments

- Determined temporal expression of Whn during early development and pregnancy
- Generated transgenic mice expressing Whn from the MMTV promoter
- Identified the expression of Whn in human breast cancer cell lines

Reportable outcomes

- Poster presentation at Massachusetts Department of Public Health Breast Cancer Research Symposium, April 13th 2000
- Generation of transgenic mice expressing Whn from the MMTV promoter

Conclusions

We have found that *whn* is expressed in the epithelial cells of the mammary gland and that expression is detected at times when the gland is actively growing and differentiating. Serum levels of estradiol and progesterone were tested and found to be comparable between nude and wild-type mice. In addition the transcription of milk proteins, which are under hormonal control, is unaffected in nude mice. These data show that the expression of Whn is directly involved in the growth and differentiation of mammary epithelial cells. Transgenic mice have been generated in which Whn is targeted to the mammary epithelium through the mouse mammary tumor virus (MMTV) promoter. These mice have been initially characterized and will be used to establish the effects of over-expression of *whn* on mammary gland development and function. Finally, Whn can be detected in cell lines derived from normal human mammary epithelia and breast tumors. The protein isolated from the mammary cell lines resolves as multiple bands by SDS-PAGE analysis, similar if not identical to the protein isolated from keratinocytes, suggesting that Whn is controled in the same way in both systems. Initial analysis indicates that in several tumor cell lines, Whn expression is lower than in a normal breast cell line, suggesting that the loss of Whn contributes to tumorigenesis of mammary epithelial cells. This is consistent with the fact that increased Whn expression in keratinocytes promotes the early stages of differentiation of the cell in which the protein is expressed. Further experiments are required to establish the significance of Whn expression levels in mammary tumor cell lines.

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Appendix 1

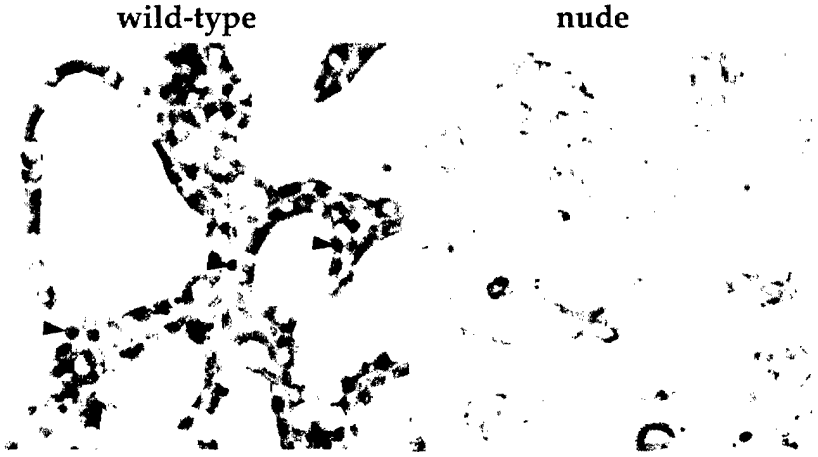

Figure 1. Whn is detected in the nuclei of mammary epithelial cells. Mammary glands were taken from nude and wild-type mice at day 18 of pregnancy, fixed in formalin and embedded in paraffin. Sections were stained by immunohistochemistry with polyclonal antibodies generated against a full length murine Whn-GST fusion protein (anti-GST-Whn) and counterstained with hematoxylin. The dark reddish brown color in the wild-type sections indicates Whn staining. Representative nuclei are indicated by arrow heads.

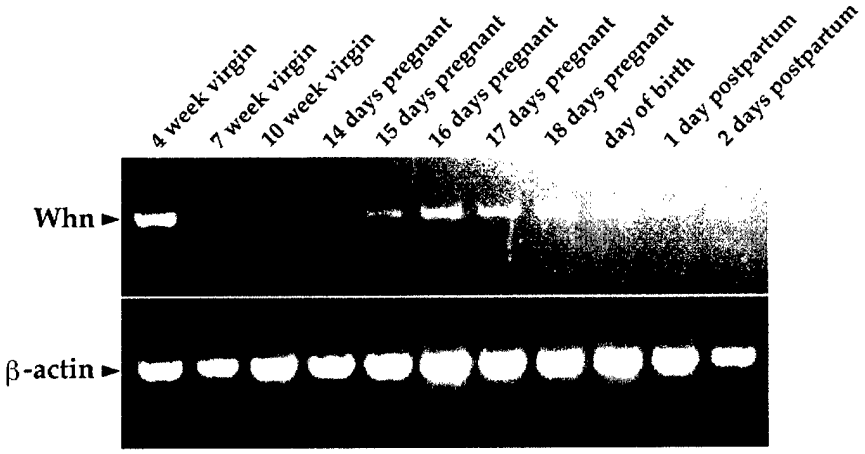

Figure 2. Whn expression is detected in developing and pregnant mammary glands. RNA was isolated from mammary glands taken from wild-type mice at the indicated times during mammary gland development and pregnancy. The presence of *whn* transcript was detected by reverse transcriptase-mediated polymerase chain reaction (RT-PCR), with primers corresponding to nucleotides 774-796 and 1449-1470 of the *whn* cDNA (Nehls et al., 1994). *Whn* transcript was detected in developing virgin mammary glands at 4 weeks, and also at all stages of pregnancy from 15 days onwards. Oligonucleotide primers corresponding to nucleotides 205-227 and 698-723 of murine *β-actin* cDNA (Tokunaga et al., 1986), a gene that is constitutively expressed, were used as a positive control.

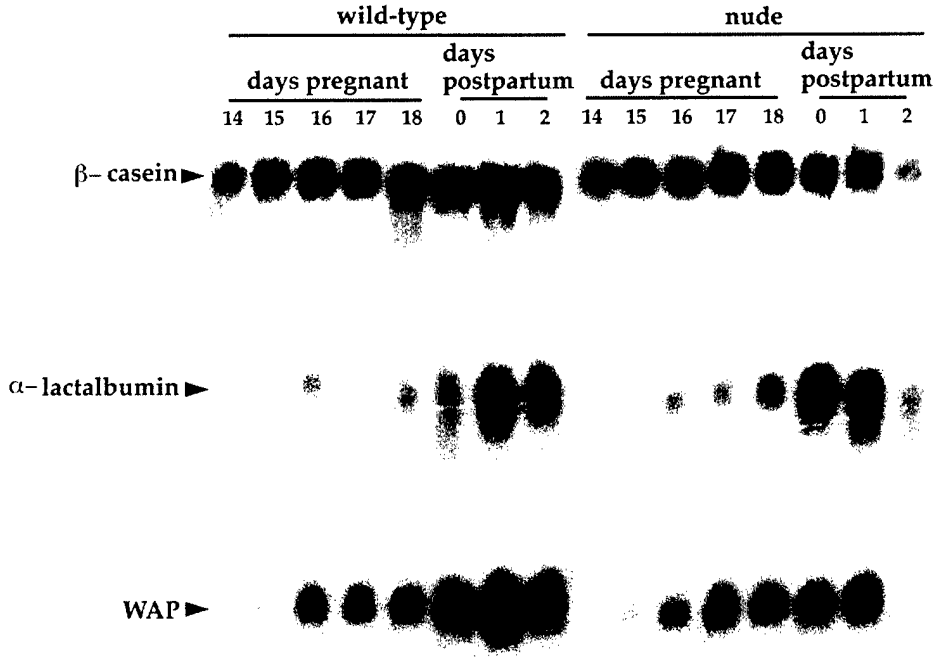

Figure 3. Milk protein gene transcription is unaffected in nude mice. Total RNA was extracted from nude and wild-type mice at developmental stages indicated. 30 μg total RNA was separated on 1.2% agarose/formaldehyde gels and transferred to Hybond N membranes. Blots were probed with ^{32}P-labeled probes corresponding to full length cDNAs for α-lactalbumin, β-casein and WAP.

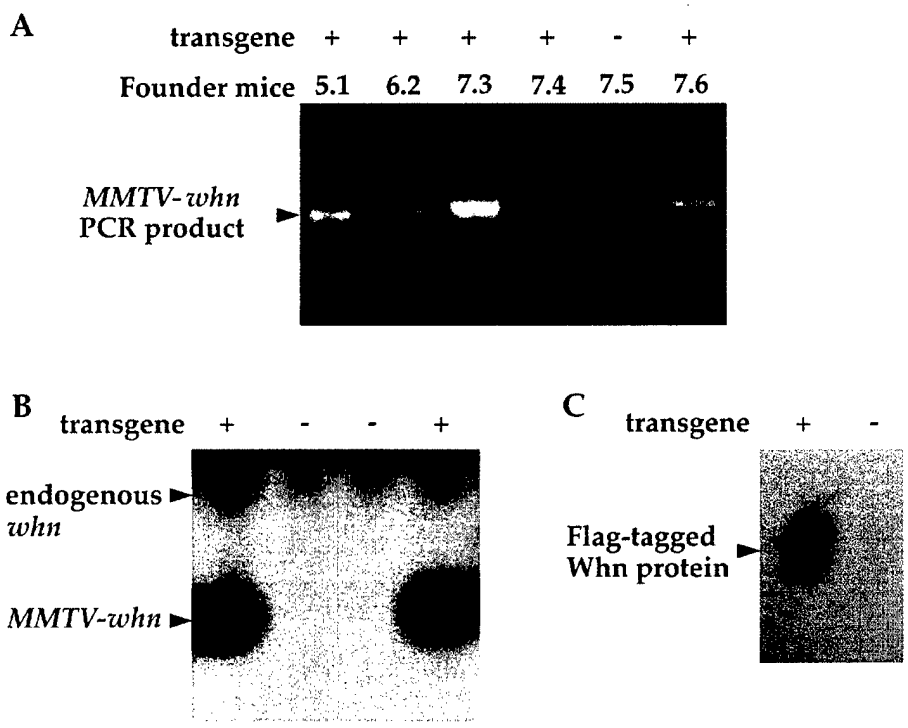

Figure 4. Characterization of *MMTV-whn* transgenic mice. A. DNA was extracted from the tails of founder mice and the presence of the *MMTV-whn* transgene detected using oligonucleotide primers corresponding to nucleotides 774-796 and 1449-1470 of the *whn* cDNA (Nehls et al., 1994). B. Genomic DNA was incubated with restriction enzymes that produce different sized fragments from the endogenous *whn* gene and the *MMTV-whn* transgene. DNA was separated on 0.8% agarose and transferred to nylon membrane. Membranes were incubated with a ^{32}P-labeled probe corresponding to nucleotides 1339-1735 of the Whn cDNA (Nehls et al., 1994). C. Mammary tissue lysates were prepared from transgenic and control mice. Lysates were incubated with antibodies to the FLAG epitope, and immune complexes recovered with protein-G sepharose, separated by SDS-PAGE and transferred to PVDF membranes. Membranes were incubated with anti-GST-Whn (1:500 dilution), and proteins were visualized using horseradish peroxidase-conjugated secondary antibodies (1:2000 dilution) and the enhanced chemiluminescence (ECL) detection system.

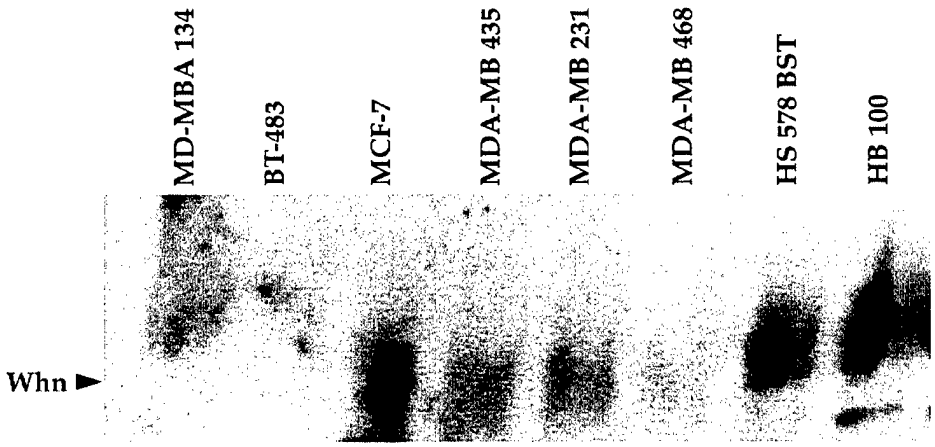

Figure 5. Whn is detected in normal human breast and breast cancer cell lines. Cells were lysed in buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM PMSF, 1 mM Na_3VO_4. Equal amounts of total protein were incubated with anti-GST-Whn antibody, and immune complexes were recovered with protein-G sepharose, separated by SDS-PAGE and transferred to PVDF membranes. Membranes were incubated with anti-GST-Whn (1:500 dilution), and proteins were visualized using horseradish peroxidase-conjugated secondary antibodies (1:2000 dilution) and the enhanced chemiluminescence (ECL) detection system.